

Supplementary Information 2

We initially evaluated two possible neuronal inputs that could result in microbiota–dependent modulation of gut sympathetic neurons: i) direct sensing of the intestinal environment by CG-SMG sympathetic neurons due to innervation of the GI tract^{1,2} and expression of Gpr41 by sympathetic neurons³; ii) changes in iEAN activity that could influence sensory afferents or sympathetic neurons directly (viscerofugal neurons)⁴⁻⁶.

i) To visualize the extent to which sympathetic innervation reaches the gut epithelium, we injected retrograde adeno-associated virus AAVrg-CAG-FLEX-tdTomato into the CG-SMG of *Snap25^{Cre}* mice, thereby restricting the expression of Tomato protein to neurons (Extended Data Fig. 5a-c). As an independent and complementary strategy, AAVrg-hSyn1-Cre was injected into the CG-SMG of *Rosa^{lsL-tdTomato}* mice (Extended Data Fig. 5d-f). In both approaches, we observed restriction of viruses to the ganglion and the immediate surrounding tissue (Extended Data Fig. 5b, d). Whole-mount immunofluorescence or cleared–tissue imaging of the distal intestine showed innervation of the two neuronal plexuses, with sparse fibres extending to the mucosa and, in rare cases, direct epithelial contact. (Extended Data Fig. 5b-f, Supplementary Video 1,2). These results indicate that sympathetic eEANS may not be positioned to directly detect signals from the epithelial layer. To more directly address direct sensing, we performed chemical sympathetic denervation using 6-OHDA. While ablation of gut sympathetic innervation per se led to activation of CG-SMG neurons, a significant increase in cFos+ sympathetic neurons was still observed post-streptomycin treatment (Extended Data Fig 5g, h). It remains plausible that signals such as butyrate diffuse through the gut tissue or act via the circulation to influence CG-SMG neurons, which express a SCFA receptor *Ffar3* (encoding Gpr41)^{3,4}, as confirmed by our TRAP-seq data (Extended Data Fig. 5i). However, systemic (intraperitoneal) administration of tributyrin or sodium butyrate (*data not shown*) instead led to robust activation of the CG-SMG (Extended Data Fig. 5j). The above data suggest that direct detection by sympathetic neurons is not the primary mechanism of microbiota–mediated suppression of CG-SMG activation.

ii) Viscerofugal neurons are thought to be activated by tissue distension and could provide excitatory or inhibitory inputs to CG-SMG neurons^{7,8}. To address whether viscerofugal neurons directly regulate microbiota–dependent CG-SMG activation, we injected fluorescent CTB into the CG-SMG and identified a sparse population of CTB⁺ intrinsic EANS (iEANS), likely viscerofugal⁷, in the intestine muscularis⁷ (Extended Data Fig. 5k). To confirm that iEANS can make synaptic contacts with sympathetic neurons, we injected iEAN-tropic AAV6-CAG-FLEX-tdTomato⁹ into the ileum of *Snap25^{Cre}* mice (Extended Data Fig. 5l). We detected tdTomato+ fibres within the CG-SMG, suggesting that viscerofugal neurons were capable

of direct communication with sympathetic neurons (Extended Data Fig. 5l). To assess a possible link between activation of iEANs and the CG-SMG, we quantified the activation state of iEANs and viscerofugal neurons upon antibiotic treatment using cFos expression^{10,11}. We injected fluorescent CTB into the CG-SMG of wild-type mice to retrograde label viscerofugal neurons. Oral gavage of streptomycin did not result in a change in the total number of neurons or viscerofugal neurons, and also did not lead to increased cFos levels in viscerofugal neurons in the ileum or distal intestine; in fact, we observed a significant decrease in cFos+ iEAN in the colon of antibiotic-treated animals (Extended Data Fig. 5m-v). We concluded that viscerofugal iEANs are likely not playing a direct role in modulation of CG-SMG neurons; however, because changes in the microbial load can rapidly affect the activation state of iEANs, it remains possible that iEANs could influence sensory neuronal pathways¹².

References

- 1 Pavlov, V. A., Chavan, S. S. & Tracey, K. J. Molecular and Functional Neuroscience in Immunity. *Annual review of immunology* **36**, 783-812, doi:10.1146/annurev-immunol-042617-053158 (2018).
- 2 Dewhirst, F. E. *et al.* Phylogeny of the defined murine microbiota: altered Schaedler flora. *Applied and environmental microbiology* **65**, 3287-3292 (1999).
- 3 Kimura, I. *et al.* Short-chain fatty acids and ketones directly regulate sympathetic nervous system via G protein-coupled receptor 41 (GPR41). *Proceedings of the National Academy of Sciences of the United States of America* **108**, 8030-8035, doi:10.1073/pnas.1016088108 (2011).
- 4 Abe, C. *et al.* C1 neurons mediate a stress-induced anti-inflammatory reflex in mice. *Nature neuroscience* **20**, 700-707, doi:10.1038/nn.4526 (2017).
- 5 Atarashi, K. *et al.* Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* **500**, 232-236, doi:10.1038/nature12331 (2013).
- 6 Biggs, M. B. *et al.* Systems-level metabolism of the altered Schaedler flora, a complete gut microbiota. *ISME J* **11**, 426-438, doi:10.1038/ismej.2016.130 (2017).
- 7 Hibberd, T. J., Zagorodnyuk, V. P., Spencer, N. J. & Brookes, S. J. Identification and mechanosensitivity of viscerofugal neurons. *Neuroscience* **225**, 118-129, doi:10.1016/j.neuroscience.2012.08.040 (2012).
- 8 Szurszewski, J. H., Ermilov, L. G. & Miller, S. M. Prevertebral ganglia and intestinofugal afferent neurones. *Gut* **51 Suppl 1**, i6-10 (2002).
- 9 Benskey, M. J. *et al.* Targeted gene delivery to the enteric nervous system using AAV: a comparison across serotypes and capsid mutants. *Molecular therapy : the journal of the American Society of Gene Therapy* **23**, 488-500, doi:10.1038/mt.2015.7 (2015).
- 10 Fung, C. *et al.* Cholinergic Submucosal Neurons Display Increased Excitability Following in Vivo Cholera Toxin Exposure in Mouse Ileum. *Front Physiol* **9**, 260, doi:10.3389/fphys.2018.00260 (2018).
- 11 Sharkey, K. A., Parr, E. J. & Keenan, C. M. Immediate-early gene expression in the inferior mesenteric ganglion and colonic myenteric plexus of the guinea pig. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **19**, 2755-2764 (1999).
- 12 Perez-Burgos, A., Mao, Y. K., Bienenstock, J. & Kunze, W. A. The gut-brain axis rewired: adding a functional vagal nicotinic "sensory synapse". *FASEB J* **28**, 3064-3074, doi:10.1096/fj.13-245282 (2014).